

## The vitamin D receptor in cancer

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DOI:

[10.1017/S0029665108006964](https://doi.org/10.1017/S0029665108006964)

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*Document Version*

Publisher's PDF, also known as Version of record

*Citation for published version (Harvard):*

Thorne, J & Campbell, M 2008, 'The vitamin D receptor in cancer', Paper presented at Proceedings of The Nutrition Society, 1/05/08 pp. 115-127. <https://doi.org/10.1017/S0029665108006964>

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The Summer Meeting of the Nutrition Society, hosted by the Irish Section, was held at the University of Ulster, Coleraine on 16–19 July 2007

## Symposium on ‘Diet and cancer’

### The vitamin D receptor in cancer

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Over the last 25 years roles have been established for vitamin D receptor (VDR) in influencing cell proliferation and differentiation. For example, murine knock-out approaches have revealed a role for the VDR in controlling mammary gland growth and function. These actions appear widespread, as the enzymes responsible for 1 $\alpha$ ,25-dihydroxycholecalciferol generation and degradation, and the VDR itself, are all functionally present in a wide range of epithelial and haematopoietic cell types. These findings, combined with epidemiological and functional data, support the concept that local, autocrine and paracrine VDR signalling exerts control over cell-fate decisions in multiple cell types. Furthermore, the recent identification of bile acid lithocholic acid as a VDR ligand underscores the environmental sensing role for the VDR. *In vitro* and *in vivo* dissection of VDR signalling in cancers (e.g. breast, prostate and colon) supports a role for targeting the VDR in either chemoprevention or chemotherapy settings. As with other potential therapeutics, it has become clear that cancer cells display *de novo* and acquired genetic and epigenetic mechanisms of resistance to these actions. Consequently, a range of experimental and clinical options are being developed to bring about more targeted actions, overcome resistance and enhance the efficacy of VDR-centred therapeutics.

**Vitamin D receptor: 1 $\alpha$ ,25-Dihydroxycholecalciferol: Prostate cancer: Breast cancer**

#### The cancer burden

The impact of cancer continues to be one of the greatest burdens in the developed world and is also increasingly impacting on the developing world. Approximately 1.5 million individuals will die from breast, colon or prostate cancer this year, and the total number of deaths from cancer accounts for 13% of all deaths worldwide and for one in every four deaths in the UK and USA<sup>(1)</sup>. The impact is also economic; \$280  $\times$  10<sup>9</sup> is spent annually worldwide on the treatment of patients. Many cancers are however preventable, and through lifestyle choices such as smoking, diet and exercise the worldwide incidence of cancer could be cut by 40%<sup>(2,3)</sup>.

#### Major risk factors for cancer

##### Diet

Recently, the appreciation of the impact of diet on cancer has come to the fore, with a number of studies establishing

unequivocal relationships between diet and cancer initiation and progression. Reflecting the accumulation of these data, the WHO has now stated that diet forms the second-most preventable cause of cancer (after smoking)<sup>(3)</sup>. This impact will rise further as a result of demographic factors, and quite possibly because of changing dietary habits worldwide, which will contribute further to the projected increase in cancer incidence in developing nations. High-profile malignancies such as breast, prostate, and colon cancer typify this scenario, in which the aetiology of the disease reflects the cumulative impact of dietary factors over an individual's lifetime<sup>(4–6)</sup>. The relationship between diet and disease is already exploited clinically, e.g. in the Selenium and Vitamin E Cancer Prevention Trial to assess the chemoprevention potential of vitamin E and Se in prostate cancer<sup>(7–9)</sup>.

Despite the importance and potential clinical benefit of these relationships it remains unclear as to what is the critical time-frame when dietary factors may be protective against cancer development, e.g. during embryogenesis,

childhood development or adult life. Resolving this issue is, understandably, highly challenging. Considerable resources were required to elucidate what is now established as a clear causal relationship between cigarette smoke and lung cancer. To address these issues the emerging field of nutrigenomics aims to dissect the impact of dietary factors on genomic regulation, and thereby physiology and pathophysiology, utilizing a range of post-genomic technologies<sup>(10)</sup>.

### *The complex aetiology of cancer*

The search for a genetic component(s) to many cancers in this post-genomic era has failed to yield significant results and only a few cancers appear to have a strong genetic component. For example, mutations in the *BRCA1* and *BRCA2* genes in breast cancer were identified in the 1990s and typically show strong penetrance with a strong familial-linked risk, but these mutations contribute to <5% of breast cancers<sup>(11,12)</sup>. A more recent study implementing genome-wide analyses has indicated five novel alleles that are common in the population and increase the risk of breast cancer, therefore suggesting a role for genetic background in the susceptibility to breast cancer<sup>(13)</sup>. A contemporary view of cancer is that there are many low-penetrance genetic factors that combine with environmental insults over the lifetime of the individual to bring about cancer. It is thought that in most cells, two insults are required to lose control of the cell cycle control<sup>(14)</sup> and between six and ten mutational events to develop into a fully-mature cancer<sup>(15)</sup>. The recent announcement of plans to immunize girls between 12 and 13 years of age against the sexually-transmitted human papillomavirus applies this theory directly to prevent an essential environmental insult required before a vital step in the transformation of normal cells to cervical cancer can occur. This vaccination programme is estimated to prevent approximately 70% of cervical cancer cases in the UK<sup>(16)</sup>. It is this complex interaction between environmental and low-penetrance genetic factors that means that age is the single biggest risk factor for the development of cancer because, simply put, there has been more time for environmental insults to impact on precancerous cells.

The sporadic temporal acquisition of a cancer phenotype is also compatible with models of disruption of the self-renewal of epithelial tissues. It has become increasingly clear that breast, colon and prostate tissues, in common with other epithelial tissues and many other cell types in the adult human subject, are self-renewing and contain committed stem cell components<sup>(17–22)</sup>. These stem cells are slowly proliferating and are able to undergo asymmetric divisions to give rise both to other stem cells and to transiently-amplifying populations of progenitor cells. The latter in turn give rise to the differentiated cell types that typify the functions of these tissues and are subsequently lost through programmed cell death processes and replaced by newly-differentiated transiently-amplifying cells. The mechanisms that control the intricate balance of these processes of division, differentiation and programmed cell death are the subjects of major investigations. These studies have revealed common roles for Wnt and hedgehog

signalling and the actions of other signal transduction processes that govern cell cycle progression, with gene targets such as the cyclin-dependent kinase inhibitor CDKN1A (p21<sup>(waf1/cip1)</sup>) emerging as points of criticality upon which numerous signal pathways converge<sup>(18–21)</sup>.

Stem cells of any tissue also have a high proliferative capacity and are the ideal candidates for tumourigenesis because they are programmed for self-renewal. It is likely to take fewer disruptions to maintain this activation than switching it on *de novo* in a more differentiated cell. Furthermore, by self-renewing, stem cells are relatively long lived compared with other cells within tissues. Although it has become apparent that there are numerous mechanisms in place in stem cells to ensure genomic integrity, the longevity of these cells results in a greater likelihood of genetic, cytogenetic or epigenetic disruptions accumulating or being passed on to daughter progenitors<sup>(23)</sup>.

Tissue self-renewal is controlled by intrinsic and extrinsic cues, including a range of intrinsic, e.g. niche signals, and extrinsic hormonal and dietary cues, which appear to regulate many of the processes associated with differentiation and programmed cell death<sup>(24,25)</sup>. The primary genomic sensor for many dietary and environmental (e.g. xenobiotic) factors is the nuclear receptor superfamily of ligand-activated transcription factors, which bind steroid hormones, vitamin micronutrients and macronutrients such as fatty acids, lipids and bile acids<sup>(26–29)</sup>.

### **The nuclear receptor superfamily**

The nuclear receptor superfamily, the largest family of transcription factors, is responsible for the sensing of hormonal, environmental and dietary-derived factors, and the translation of these signals into appropriate transcriptional responses<sup>(30–35)</sup>. Often working co-operatively, nuclear receptors converge on common gene targets to give tight regulation of gene expression and repression. Thus, nuclear receptors integrate dietary extracellular signals into cell-fate decisions such as cell cycle control, self-renewal and xenobiotic clearance.

### *Structure and function*

A broad classification of the nuclear receptor superfamily can be outlined according to ligand affinities. The first group of receptors, exemplified by sex steroid and thyroid hormone receptors, binds ligands with high affinity. A number of nutrient-derived ligands are also bound with high affinity by specific receptors. For example, 1 $\alpha$ ,25-dihydroxycholecalciferol (1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>) and the retinoids (all-*trans*- and 9-*cis*-retinoic acid) are bound by the vitamin D receptor (VDR) and by the retinoic acid and retinoid X receptors respectively. The second group of receptors, e.g. the PPAR, liver X receptors and farnesoid X receptor, bind with broader affinity more-abundant lipophilic compounds such as macronutrients, PUFA and bile acids. Finally, a group of orphan receptors exists, which either has no functional ligand-binding domain or no ligands have been identified as yet. By contrast, phylogenetic classification has defined seven subfamilies, the VDR

being in the group 1 subfamily, sharing homology with the liver X receptors and farnesoid X receptor and more distantly the PPAR<sup>(36,37)</sup>.

The nuclear receptors share a common architecture, which includes defined regions for DNA recognition, ligand binding and cofactor interactions. The DNA-binding domain recognizes specific response elements (RE), which were originally characterized in the enhancer–promoter regions of target genes. More recently, such functionally-responsive regions have been characterized in both intronic and 3' regions and gene regulation is brought about through the coordinated actions in multiple responsive regions<sup>(18,38)</sup>. Most receptors preferentially form homo- or heterodimeric complexes; retinoid X receptor is a central partner for VDR, PPAR, liver X receptors and farnesoid X receptor. Thus, simple RE are formed by two recognition motives and their relative distance and orientation contributes to receptor binding specificity, although more recently larger, composite and integrated elements have been identified, suggesting a more intricate control<sup>(23,39,40)</sup>.

### The vitamin D receptor

#### *Metabolism of cholecalciferol and major cholecalciferol functions*

Systemic monitoring and regulation of serum Ca levels are fundamentally important processes because of the vital function that Ca plays in a wide range of cellular functions. The VDR plays a well-established endocrine role in the regulation of Ca homeostasis, in particular by regulating Ca absorption in the gut and regulating bone mineralization<sup>(41–43)</sup>. In turn,  $1\alpha,25(\text{OH})_2\text{D}_3$  status is dependent on cutaneous synthesis initiated by solar radiation and also on dietary intake; a reduction in one or both sources leads to vitamin D insufficiency. Interestingly, the contribution from the UV-initiated cutaneous conversion of 7-dehydrocholesterol to vitamin D is the greater, contributing >90% towards  $1\alpha,25(\text{OH})_2\text{D}_3$  synthesis in a vitamin D-sufficient individual<sup>(44)</sup>. The importance of the relationships between solar exposure and the ability to capture UV-mediated energy is underscored by the inverse correlation between human skin pigmentation and latitude; i.e. the individual capacity to generate  $1\alpha,25(\text{OH})_2\text{D}_3$  in response to solar UV exposure is intimately associated with forebear environmental adaptation<sup>(44)</sup>. The correct and sufficient level of solar exposure and serum vitamin D are matters of considerable debate. Current recommendations for daily vitamin D intake are in the range of 10–20 µg/d. More recently, reassessment of the  $1\alpha,25(\text{OH})_2\text{D}_3$  impact on the prevention of osteoporosis has suggested that the correct level may be as high as 50–75 µg/d<sup>(45)</sup>, which may reflect more accurately 'ancestral' serum levels.

The importance of the relationship between UV exposure and Ca homeostasis has been understood for >100 years and has driven the endocrine view of  $1\alpha,25(\text{OH})_2\text{D}_3$  signalling with spatially-distinct sites within the body of incremental vitamin D activation. Thus, vitamin D produced in the skin is converted in the liver to 25-hydroxycholecalciferol, (25OH-D), and circulating levels of this metabolite serve as a useful index of vitamin D status.

A further hydroxylation occurs in the kidney at the C-1 position by 25-hydroxyvitamin D-1 $\alpha$ -hydroxylase (encoded by *CYP27b1*) to produce the biologically-active hormone  $1\alpha,25(\text{OH})_2\text{D}_3$ <sup>(44)</sup>. A second mitochondrial cytochrome P450 enzyme, the 24-hydroxylase (encoded by *CYP24*) enzyme, can utilize both 25OH-D and  $1\alpha,25(\text{OH})_2\text{D}_3$  as substrates and is the first step in the inactivation pathway for these metabolites.

More recently, the expression of the 25OH-D activating enzyme, *CYP27b1*, has been identified in keratinocytes and a wide range of other cell types. In parallel, an autocrine–paracrine role for the local synthesis and signalling of  $1\alpha,25(\text{OH})_2\text{D}_3$  has been uncovered<sup>(46–51)</sup>. Thus, in multiple target tissues 25OH-D may enter into an intracellular VDR signalling axis that coordinates the local synthesis, metabolism and signal transduction of  $1\alpha,25(\text{OH})_2\text{D}_3$ . The components of this axis have been shown to be regulated dynamically, as *CYP27b1* is repressed by  $1\alpha,25(\text{OH})_2\text{D}_3$  and correspondingly *CYP24* is positively regulated by  $1\alpha,25(\text{OH})_2\text{D}_3$ . Thus, elevated levels of  $1\alpha,25(\text{OH})_2\text{D}_3$  appear to block its synthesis and induce its own inactivation<sup>(52)</sup> in a classical negative-feedback loop. The ability of the VDR to play roles in both transactivation and trans-repression reflects emerging themes for other nuclear receptors, e.g. PPAR<sup>(53,54)</sup>, and suggests a hitherto unsuspected flexibility of the VDR to associate with a diverse array of protein factors to adapt function<sup>(55,56)</sup>. The biological importance of these autocrine actions have been the subject of intense investigation, and support the concept that the VDR has two, perhaps distinct, broad biological roles, i.e. the endocrine regulation of serum Ca and the autocrine–paracrine regulation of biological functions associated with the regulation of cell proliferation and differentiation and with the modulation of immune responses.

#### *Apo and Holo nuclear receptor states*

A current challenge in nuclear receptor biology, and especially pertinent for the VDR, is to define mechanisms that modulate and limit the transcriptional potential, and bring about promoter targeting specificity. Expression, localization and isoform composition of co-repressor complexes have emerged as important determinants of the spatio-temporal equilibrium point between the antagonistic actions of the *apo* and *holo* nuclear receptor complexes, and consequently target gene promoter responsiveness<sup>(34,57–65)</sup>.

Efforts to understand nuclear receptor function have at their basis the antagonism between these *apo* and *holo* nuclear receptor complexes, a direct effect of which is the regulation of a diverse range of histone modifications. Histone modifications at the level of meta-chromatin architecture appear to form a stable and heritable 'histone code', such as in X chromosome inactivation (for review, see Turner<sup>(66)</sup>). The extent to which similar processes operate to govern the activity of micro-chromatin contexts, such as gene promoter regions, is an area of debate<sup>(67,68)</sup>. The *apo* and *holo* nuclear receptor complexes initiate specific and coordinated histone modifications<sup>(69,70)</sup> to govern transcriptional responsiveness of the promoter. There is



good evidence that specific histone modifications also determine the assembly of transcription factors on the promoter and control individual promoter transcriptional responsiveness<sup>(71–73)</sup>. It is less clear to what extent nuclear receptors recognize basal histone modifications on target gene promoters; functional studies of the SANT motif contained in the co-repressor NCoR2/SMRT support this latter idea<sup>(74)</sup>. This area is complex and rapidly evolving (for an excellent recent review, see Rosenfeld *et al.*<sup>(53)</sup>).

In the absence of ligand VDR–retinoid X receptor dimers exist in an ‘apo’ state, as part of large complexes (approximately 2.0 MDa)<sup>(75)</sup>, associated with co-repressors (e.g. NCoR2/SMRT) and bound to RE sequences. These complexes actively recruit a range of enzymes that post-translationally modify histone tails, e.g. histone deacetylases and methyltransferases, and thereby maintain a locally condensed chromatin structure around RE sequences<sup>(29)</sup>. Ligand binding induces a so-called *holo* state, facilitating the association of the VDR–retinoid X receptor dimer with co-activator complexes. A large number of interacting co-activator proteins, which can be divided into multiple families including the p160 family, the non-p160 members and members of the large ‘bridging’ DRIP/TRAP/ARC complex, have been described that link the receptor complex to the co-integrators CBP/p300 and basal transcriptional machinery<sup>(26,45,76,77)</sup>. These receptor–co-activator complexes coordinate the activation of an antagonistic battery of enzymes, such as histone acetyltransferases, and thereby induce the reorganization of local chromatin regions at the RE of the target gene promoter. The complex choreography of this event has recently emerged and involves cyclical rounds of promoter-specific complex assembly, gene transactivation, complex disassembly and proteasome-mediated receptor degradation<sup>(26,45,78)</sup>.

The expression, localization and isoforms of co-repressor complexes have emerged as critical in determining the spatio-temporal equilibrium between the antagonistic actions of the *apo* and *holo* nuclear receptor complexes, and thus determine target gene promoter responsiveness in a range of physiological and pathological settings<sup>(79–81)</sup>.

## VDR and cancer

### *Evidence of vitamin D receptor involvement in cancer*

In 1981  $1\alpha,25(\text{OH})_2\text{D}_3$  was shown to inhibit human melanoma cell proliferation significantly *in vitro* at nanomolar concentrations<sup>(82)</sup> and was subsequently found to induce differentiation in cultured mouse and human myeloid leukaemia cells<sup>(83,84)</sup>. Following these studies anti-proliferative effects have been demonstrated in a wide variety of cancer cell lines, including those from the prostate and breast<sup>(85–92)</sup>. Thus, common models of VDR responses include MCF-7 breast cancer cells, LNCaP prostate cancer cells and CaCo2 colon cancer cells.

In order to identify critical target genes that mediate these actions, comprehensive genome-wide *in silico* and transcriptomic screens have analysed the anti-proliferative VDR transcriptome and revealed broad consensus on certain targets, but has also highlighted variability<sup>(85,93–95)</sup>.

This heterogeneity may in part reflect experimental conditions, cell line differences and genuine tissue-specific differences in cofactor expression that alter the magnitude and extent of VDR transcriptional actions. The common anti-proliferative VDR functions are associated with arrest at G<sub>0</sub>/G<sub>1</sub> of the cell cycle, coupled with up-regulation of a number of cell cycle inhibitors including p21<sup>(waf1/cip1)</sup> and p27<sup>(kip1)</sup>. Promoter characterization studies have demonstrated a series of vitamin D-responsive elements in the promoter–enhancer region of *CDKN1A*, a primary  $1\alpha,25(\text{OH})_2\text{D}_3$ -responding gene<sup>(96,97)</sup>. By contrast, p27<sup>(kip1)</sup> protein levels appear to be regulated by a range of post-transcriptional mechanisms, such as enhanced mRNA translation, and attenuating degradative mechanisms, often in a cell-type-specific manner<sup>(98–100)</sup>. The up-regulation of p21<sup>(waf1/cip1)</sup> and p27<sup>(kip1)</sup> principally mediate G<sub>1</sub> cell cycle arrest, but  $1\alpha,25(\text{OH})_2\text{D}_3$  has been shown to mediate a G<sub>2</sub>/M cell cycle arrest in a number of cancer cell lines via direct induction of *GADD45* $\alpha$ <sup>(94,101,102)</sup>. Again, this regulation appears to combine direct gene transcription and a range of post-transcriptional mechanisms. These studies highlight the difficulty of establishing strict transcriptional effects of the VDR, as a range of post-transcriptional effects act in concert to regulate target protein levels. Another VDR effect is associated with elevated expression of a number of brush-border-associated enzymes such as alkaline phosphatase, as well as intermediate filaments, vinculin, ZO-1, ZO-2, desmosomes and E-cadherin, which collectively enhance adhesion and suppress migration<sup>(103)</sup>.

Another VDR action, notably in MCF-7 breast cancer cells, is a profound and rapid induction of apoptosis, irrespective of p53 content, which may reflect the VDR role in the involution of the post-lactating mammary gland. The direct transcriptional targets that regulate these actions remain elusive, although there is growing evidence of an involvement of the *BAX* family of proteins<sup>(104,105)</sup>. Induction of programmed cell death following  $1\alpha,25(\text{OH})_2\text{D}_3$  treatment is also associated with increased generation of reactive oxygen species.  $1\alpha,25(\text{OH})_2\text{D}_3$  treatment up-regulates *VDUP1* encoding vitamin D up-regulated protein 1, which binds to the disulfide-reducing protein thioredoxin and inhibits its ability to neutralize reactive oxygen species, thereby potentiating stress-induced apoptosis<sup>(106,107)</sup>. In other cells the apoptotic response is delayed and not so pronounced, probably reflecting less-direct effects. Taken together, these data suggest that the extent and timing of apoptotic events depend on the integration of VDR signalling with other cell signalling systems.

### *Epidemiological evidence*

Epidemiological studies by Garland and associates have demonstrated that intensity of local sunlight is inversely correlated with risk of certain cancers including breast, prostatic and colo-rectal carcinoma<sup>(108–113)</sup>. Supportively, levels of 25OH-D, the major circulating metabolite of vitamin D, are significantly lower in patients with breast cancer than in age-matched controls<sup>(114)</sup>. Furthermore, there are reduced *CYP27b1* mRNA and protein levels in breast cancer cell lines and primary tumours<sup>(115)</sup>.

Comparative genome hybridization studies have found that *CYP24* is amplified in human breast cancer and *CYP24* is associated with altered patterns of  $1\alpha,25(\text{OH})_2\text{D}_3$  metabolism<sup>(51,116)</sup>. Thus, over-expression of 24-hydroxylase may further abrogate growth control mediated by  $1\alpha,25(\text{OH})_2\text{D}_3$ , via target cell inactivation of the hormone. It has therefore been proposed that breast cancer is associated with low circulating concentrations of 25OH-D, arising as a result of reduced exposure to sunlight, altered dietary patterns and impaired generation of  $1\alpha,25(\text{OH})_2\text{D}_3$  within breast tissue<sup>(51,117–121)</sup>.

Parallel epidemiological studies have also linked the incidence of prostate cancer to vitamin D insufficiency as a result of either diet or environment. In 1990 Schwartz and colleagues suggested a role for vitamin D in decreasing the risk for prostate cancer based on the observation that mortality rates in the USA are inversely related to incident solar radiation<sup>(112)</sup>. Recently, a study of men in the San Francisco Bay area has reported a reduced risk of advanced prostate cancer associated with high sun exposure, and similar relationships have been established in UK populations<sup>(110,122)</sup>. As with breast cancer, the proposed mechanism for the protective effects of sunlight on prostate risk involves the local generation of  $1\alpha,25(\text{OH})_2\text{D}_3$  from circulating 25OH-D in prostate epithelial cells. Cancerous prostate cells express reduced  $1\alpha$ -hydroxylase activity. Prediagnostic serum levels of 25OH-D have been assessed in several prospective studies, with some reporting increased risk among men with low circulating levels of the vitamin D metabolite and a suggestion of an inverse relationship with advanced disease<sup>(113,118–120)</sup>.

As with breast and prostate cancer, some epidemiological studies have noted that colon cancer risk and mortality increase with increasing latitude; for example, adjusted death rates from colon cancer in Caucasian males in the USA are nearly three times higher in north eastern states than in sunnier more southerly states<sup>(123)</sup>.

#### In vivo studies

*Vitamin D receptor-knock-out mice show increased sensitivity to carcinogen challenge.* *Vdr*-deficient mice have become extremely useful tools in elucidating more clearly the role for the VDR to act in a chemopreventive manner. A series of mice have been generated in which the VDR-ablated background has been crossed into different tumour disposition phenotypes. Thus, crossing the *vdr*-deficient and heterozygote mice with mouse mammary tumour virus-*neu* transgenic mice has generated animals that show an extent of VDR haplo-sufficiency. The mammary tumour burden in the crossed mice is reduced by the presence of one wild-type *vdr* allele, and further by two wild-type *vdr* alleles<sup>(124)</sup>. In addition, *vdr*-/- mice demonstrate greater susceptibility to carcinogen challenge. For example, treatment of these mice with dimethylbenzanthracene induces more pre-neoplastic lesions in the mammary glands than in wild-type mice<sup>(125)</sup>.

*Dietary-derived cholecalciferol inhibits tumour progression.* A parallel and larger series of studies has examined the ability of dietary or pharmacological addition of vitamin D compounds either to prevent tumour

formation<sup>(126)</sup> or to inhibit growth of transplanted tumour xenografts<sup>(127,128)</sup>. Focusing on dietary regimens that demonstrate tumour predisposition, long-term studies on mice fed a Western-style diet (e.g. high fat and phosphate and low vitamin D and Ca content) have shown increased colonic epithelial cell hyperproliferation. Acute exposure to these diets, e.g. over 12 weeks, has proved sufficient to induce colon-crypt hyperplasia; effects that could be ameliorated by the addition of Ca and vitamin D<sup>(129)</sup>.

Another important model to test chemoprevention and chemotherapy is the *Apc<sub>min</sub>* mouse. APC is a key negative regulator of  $\beta$ -catenin actions and is commonly disrupted in human subjects developing colon cancer. The rate of polyp formation in *Apc<sub>min</sub>* mice is increased in mice fed a Western diet compared with animals on standard chow. Only moderate effects of  $1\alpha,25(\text{OH})_2\text{D}_3$  on polyp formation are found in this model, associated with marked hypercalcaemia. However, the effects are more pronounced and significant when a potent analogue of  $1\alpha,25(\text{OH})_2\text{D}_3$  is used, which also displays reduced toxicity<sup>(130)</sup>.

The efficacy of  $1\alpha,25(\text{OH})_2\text{D}_3$  and its analogues has also been extensively tested in carcinogen-induced models *in vivo*, indicating a range of protective effects against both tumour initiation, progression and invasion, and supporting VDR chemoprevention and chemotherapy applications. In addition, immunodeficient mice injected with human breast and other cancer cell lines show tumour growth suppression and reduced angiogenesis in response to  $1\alpha,25(\text{OH})_2\text{D}_3$ <sup>(131,132)</sup>.

*Interaction between dietary components.* A complementary approach to these studies has examined the capacity of  $1\alpha,25(\text{OH})_2\text{D}_3$  to interact with other dietary components, which are known to be chemoprotective. One such strategy has focused on the ability to enhance local autocrine synthesis and signalling of  $1\alpha,25(\text{OH})_2\text{D}_3$ . For example, phyto-oestrogens, such as genestein or those in soyabean meal, are known to be protective, and *in vivo* feeding of these substances appears to increase *CYP27B1* and reduce *CYP24* expression in the mouse colon, resulting in locally-elevated levels of  $1\alpha,25(\text{OH})_2\text{D}_3$ <sup>(133)</sup>. These results would support the concept that Asian diets, rich in phyto-oestrogens and vitamin D, may in part explain the traditionally low rates of breast, prostate and colon cancer in this region.

#### The vitamin D receptor in DNA damage and repair

The role of vitamin D in the skin is also suggestive of its chemopreventive effects. UV light from sun exposure has several effects in the skin; UVA light induces DNA damage through increasing the level of reactive oxygen species, but importantly UVB light also catalyses the conversion of 7-dehydroxycholesterol to 25OH-D and induces the expression of VDR.

Several lines of evidence suggest that vitamin D may be protective of solar-induced DNA damage. The anti-proliferative *p21<sup>(waf1/cip1)</sup>* and *GADD45 $\alpha$*  genes are direct targets of both VDR and the tumour suppressor p53. In fact, at least two VDR and p53 RE that lie within the promoter and enhancer regions of *p21<sup>(waf1/cip1)</sup>* are so closely localized that functional interaction between

promoter-bound VDR and p53 may be possible<sup>(97)</sup>. Cooperation between the VDR and p53 may therefore be vital in mediating cell cycle arrest and the repair of DNA within cells with solar and other types of DNA damage.

In addition, antimicrobial and anti-inflammatory genes are another subset of VDR targets that are induced by UV radiation. Suppression of the adaptive inflammatory response is thought to be protective for several reasons; inflamed tissues contain more reactive oxygen species that can damage DNA and prevent proper function of DNA repair machinery, also the induction of cytokines and growth factors associated with inflammation act to increase the proliferative potential of the cells. NF- $\kappa$ B is a key mediator of inflammation and the VDR attenuates this process by negatively regulating NF- $\kappa$ B signalling<sup>(134)</sup>. This control by VDR is underscored by studies showing that *vdr*<sup>-/-</sup> mice are more sensitive to chemicals that induce inflammation than their wild-types counterparts<sup>(135)</sup>. The normally protective effect of inflammation that occurs under other conditions is lost through this VDR-mediated suppression but is compensated by the induction of a cohort of antimicrobial and antifungal genes via the innate immune response<sup>(136–138)</sup>. The induction of antimicrobials not only prevents infection in damaged tissue but can be cytotoxic for cells with increased levels of anion phospholipids within their membranes, a common feature of transformed cells<sup>(139)</sup>; experimental results are, however, conflicting. Antimicrobials such as DCDMNQ show potent anti-proliferative effects in prostate cancer cells lines such as PC-3 and Du-145<sup>(140)</sup> and derivatives of 1,2,4-triazole are cytotoxic against some colon and breast cancer cell lines<sup>(141)</sup>. However, the direct VDR target LL-37, also a potent antimicrobial, appears to promote cellular proliferation in HaCaT cells<sup>(142)</sup>.

Combined, these epidemiological, *in vivo* and cell line studies have supported the clinical evaluation of vitamin D compounds in a range of cancer settings. Recent high-dose and combination clinical trials targeting the VDR in prostate cancer have proved encouraging and continue to support therapeutic exploitation of this receptor<sup>(143–146)</sup>. The proposed chemoprotective role of the VDR in the skin in terms of its interactions with p53, the suppression of inflammation and promotion of innate immune responses underscores the importance of vitamin D compounds in the prevention of cancer as well as providing a novel therapeutic target.

### Mechanisms of disruption

A major limitation in the therapeutic exploitation of  $1\alpha,25(\text{OH})_2\text{D}_3$  in cancer therapies is the resistance of cancer cells towards  $1\alpha,25(\text{OH})_2\text{D}_3$ , as transformed cell lines often display a spectrum of sensitivities including complete insensitivity to  $1\alpha,25(\text{OH})_2\text{D}_3$ , irrespective of VDR expression. One research focus to overcome this limitation has been to develop analogues of  $1\alpha,25(\text{OH})_2\text{D}_3$ . Multiple studies have demonstrated that these compounds have some enhanced potency, but resistance remains an issue. Further information about these analogues and their

uses can be found in the excellent review by Stein & Wark<sup>(147)</sup>. The VDR is neither commonly mutated nor is there a clear relationship between VDR expression and growth inhibition by  $1\alpha,25(\text{OH})_2\text{D}_3$ <sup>(148)</sup>. The molecular mechanisms for  $1\alpha,25(\text{OH})_2\text{D}_3$  insensitivity in cancer are, however, emerging.

### Genetic resistance

The gene encoding the VDR protein is known to display polymorphic variation. Thus, polymorphisms in the 3' and 5' regions of the gene have been described and variously associated with risk of breast, prostate and colon cancer, although the functional consequences remain to be established clearly<sup>(149–155)</sup>. For example, a start codon polymorphism in exon II at the 5' end of the gene, determined using the *fok*-I restriction enzyme, results in a truncated protein<sup>(156,157)</sup>. At the 3' end of the gene three polymorphisms have been identified that do not lead to any change in either the transcribed mRNA or the translated protein. The first two sequences generate *Bsm*I and *Apa*I restriction sites and are intronic, lying between exons 8 and 9. The third polymorphism, which generates a *Taq*I restriction site, lies in exon 9 and leads to a silent codon change (from ATT to ATC), either of which insert an isoleucine residue at position 352. These three polymorphisms are linked to a further gene variation, a variable-length adenosine sequence within the 3' untranslated region. The poly(A) sequence varies in length and can be segregated into two groups: long sequences of eighteen to twenty-four adenosines; short sequences<sup>(113,158–160)</sup>. The length of the poly(A) tail can determine mRNA stability<sup>(161–163)</sup>, so the polymorphisms resulting in long poly(A) tails may increase the local levels of the VDR protein.

Multiple studies have addressed the association between VDR genotype and cancer risk and progression. In breast cancer the *Apa*I polymorphism shows an association with breast cancer risk, as indeed have the *Bsm*I and the long-sequence poly(A) variant. Similarly, the *Apa*I polymorphism is associated with metastases to bone<sup>(164,165)</sup>. The functional consequences of the *Bsm*I, *Apa*I and *Taq*I polymorphisms are unclear but because of genetic linkage may act as a marker for the poly(A) sequence within the 3' untranslated region, which in turn determine transcript stability. Interestingly, combined polymorphisms and serum 25OH-D levels have been shown to further compound breast cancer risk and disease severity<sup>(166)</sup>.

Earlier studies have suggested that polymorphisms in the VDR gene might also be associated with risk of prostate cancer. Ntais and co-workers have performed a meta-analysis of fourteen published studies with four common gene polymorphisms (*Taq*I, poly(A) repeat, *Bsm*I and *Fok*I) in individuals of European, Asian and African descent. They have concluded that these polymorphisms are unlikely to be major determinants of susceptibility to prostate cancer on a wide population basis<sup>(167)</sup>. Equally, studies in colon cancer have yet to reveal conclusive relationships and may be dependent on the ethnicity of the population studied.



### Epigenetic resistance

To date no cytogenetic abnormalities of the *VDR* have been reported. Thus, exploration of epigenetic mechanisms that disrupt *VDR* signalling is being undertaken by the authors and by other groups. The lack of an anti-proliferative response is reflected by a suppression of the transcriptional responsiveness of anti-proliferative target genes such as *p21<sup>(waf1/cip1)</sup>*, *p27<sup>(kip1)</sup>*, *GADD45α* and *BRCA1*<sup>(87,102,168,169)</sup>. Paradoxically, *VDR* transactivation is sustained or even enhanced, as measured by induction of the highly  $1\alpha,25(\text{OH})_2\text{D}_3$ -inducible *CYP24* gene<sup>(170,171)</sup>. Together these data suggest that the *VDR* transcriptome is skewed in cancer cells to disfavour anti-proliferative target genes, and that lack of functional *VDR* alone cannot explain resistance. It has been proposed that apparent  $1\alpha,25(\text{OH})_2\text{D}_3$  insensitivity is the result of epigenetic events that skew the promoter responsiveness to suppress responsiveness of specific target gene promoters<sup>(172,173)</sup>.

In support, frequently elevated co-repressor mRNA expression has been found, most commonly involving *NCoR2/SMRT*, in malignant prostate primary cultures and cell lines, with reduced  $1\alpha,25(\text{OH})_2\text{D}_3$  anti-proliferative response<sup>(81,87,169,174)</sup>. These data indicate that the *VDR*: co-repressor maybe critical in determining  $1\alpha,25(\text{OH})_2\text{D}_3$  responsiveness in cancer cells. It has been reasoned that this molecular lesion could be targeted by co-treatment of ligand ( $1\alpha,25(\text{OH})_2\text{D}_3$ ) plus the histone deacetylase inhibitors such as trichostatin A. These approaches restore the  $1\alpha,25(\text{OH})_2\text{D}_3$  response of the androgen-independent PC-3 cells to levels indistinguishable from those of control normal prostate epithelial cells. This reversal of  $1\alpha,25(\text{OH})_2\text{D}_3$  insensitivity is associated with re-expression of gene targets associated with the control of proliferation and induction of apoptosis, notably *GADD45α*. A small interfering RNA approach towards *NCoR2/SMRT* has demonstrated the important role this co-repressor plays in regulating this response, with its repression resulting in profound enhancement of the induction of *GADD45α* in response to  $1\alpha,25(\text{OH})_2\text{D}_3$ . These data support a central role for elevated *NCoR2/SMRT* levels to suppress the induction of key target genes, resulting in loss of sensitivity to the anti-proliferative action of  $1\alpha,25(\text{OH})_2\text{D}_3$ <sup>(81,87,169)</sup>.

In parallel studies a similar spectrum of reduced  $1\alpha,25(\text{OH})_2\text{D}_3$  responsiveness between non-malignant breast epithelial cells and breast cancer cell lines has been demonstrated<sup>(172,175)</sup>. Again, this reduction is not determined entirely by a linear relationship between the levels of  $1\alpha,25(\text{OH})_2\text{D}_3$  and *VDR* mRNA expression. Rather, elevated co-repressor mRNA levels, notably *NCoR1*, in oestrogen receptor  $\alpha$ -negative breast cancer cell lines and primary cultures are associated with  $1\alpha,25(\text{OH})_2\text{D}_3$  insensitivity. Again targeting this molecular lesion through co-treatments of  $1\alpha,25(\text{OH})_2\text{D}_3$  with histone deacetylases inhibitors coordinately regulates *VDR* targets such as *p21<sup>(waf1/cip1)</sup>* and *GADD45α* and restores anti-proliferative responsiveness<sup>(172,175)</sup>.

Together these data support the concept that altered patterns of co-repressors inappropriately sustains histone

deacetylation around the vitamin D-responsive element of target gene promoter–enhancer regions, and shifts the dynamic equilibrium between *apo* and *holo* receptor conformations to favour transcriptional repression of key target genes such as *p21<sup>(waf1/cip1)</sup>* or *GADD45α*. Thus, *VDR* gene targets are less responsive in  $1\alpha,25(\text{OH})_2\text{D}_3$ -insensitive cancer cells compared with non-malignant counterparts. Furthermore, targeting this molecular lesion with co-treatments of cholecalciferol compounds plus histone deacetylases inhibitors generates a temporal window in which the equilibrium point between *apo* and *holo* complexes is shifted to favour a more transcriptionally permissive environment.

These findings complement a number of parallel studies undertaken by other groups, which have established cooperation between  $1\alpha,25(\text{OH})_2\text{D}_3$  and butyrate compounds, such as sodium butyrate<sup>(176–181)</sup>. These compounds are SCFA produced during fermentation by endogenous intestinal bacteria and have the capacity to act as histone deacetylases inhibitors. Stein and co-workers have identified the effects in colon cancer cells of  $1\alpha,25(\text{OH})_2\text{D}_3$  + sodium butyrate co-treatments to include the coordinate regulation of the *VDR* itself. The authors' studies, in the time-frame studied (0–24 h), have shown no evidence for changes in *VDR* mRNA levels on co-treatment with  $1\alpha,25(\text{OH})_2\text{D}_3$  plus trichostatin A. However, together these studies underscore further the importance of the dietary-derived milieu in the regulation of epithelial proliferation and differentiation beyond sites of action in the gut.

### Future therapeutic goals

These studies are a move towards chemoprevention applications and reflect the emerging appreciation of the impact of diet on either the initiation or progression of cancer and other aging syndromes. A simple preventative therapeutic measure may involve the supplementation of staple foods with vitamin D. Similar measures have been successfully implemented in the USA through adding folic acid to bread in response to the need for pregnant women to increase their intake, and in the UK through increasing *n*-3 PUFA levels in eggs by altering the composition of chicken feed.

For 'next generation' developments to occur, however, it will be necessary to adopt a broader view of *VDR* signalling. Historically, researchers have studied the abilities of single nuclear receptors such as the *VDR* to regulate a discrete group of gene targets and influence cell function. This approach has led to substantial knowledge concerning many of these receptors individually. Cell and organism function, however, depends on the dynamic interactions of a collection of receptors through the networks that link them and against the backdrop of intrinsic cellular programmes such as those governing development and differentiation. The current lack of an integral view as to how these interactions bring about function and dysfunction, e.g. in the aging human individual, can be attributed to the limitations of previously available techniques and tools to undertake such studies. The implementation of post-genomic techniques together with bioinformatics and systems biology methodology is expected to generate an integral view, thereby revealing and quantifying the mechanisms



by which cells, tissues and organisms interact with environmental factors such as diet<sup>(182,183)</sup>.

Thus, it is probably naive to assume that the VDR alone plays a key and dominant role in cell and tissue function by acting singularly, but instead is intimately linked to the actions of related nuclear receptors (e.g. PPAR, farnesoid X receptor and liver X receptors) and cofactors. Equally, the concept favoured is that the diverse signalling capacity, which appears in the skin, is retained in most cell types and reflects a combination of VDR function and its interactions with intrinsic transcriptional programmes such as self-renewal or geno-protection via p53.

The challenge is to model the spatio-temporal actions of the nuclear receptor network and, in particular, the extent to which the VDR exerts critical control over transcription and translation. Such an understanding requires a clear awareness of the chromatin architecture and context of the promoter regions (e.g. histone modifications, DNA methylation), genomic organization, gene regulation hierarchies and  $1\alpha,25(\text{OH})_2\text{D}_3$ -based metabolomic cascades, all within the context of specific cell backgrounds. The ultimate therapeutic goal will be to translate this understanding to strategies whereby only subsets of VDR actions are targeted in discrete disease settings.

### Acknowledgments

The authors gratefully acknowledge support from the BBSRC and of NucSys, a European Community FP6-funded consortium aimed at the dissection and mathematical modelling of nuclear receptor responses to nutritional signals in health and disease.

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